PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

# PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>10 August 2000 (10.08.00) | |
| **International application No.**<br>PCT/GB99/04352 | **Applicant's or agent's file reference**<br>N.78329A |
| **International filing date** (day/month/year)<br>22 December 1999 (22.12.99) | **Priority date** (day/month/year)<br>22 December 1998 (22.12.98) |
| **Applicant**<br>ERRINGTON, Jeffrey | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 July 2000 (14.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|---|---|
| **The International Bureau of WIPO**<br>**34, chemin des Colombettes**<br>**1211 Geneva 20, Switzerland** | Authorized officer<br>Pascal Piriou |
| Facsimile No.: (41-22) 740.14.35 | Telephone No.: (41-22) 338.83.38 |

PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference | **FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. | |
|---|---|---|
| N.78329A | | |
| International application No. | International filing date *(day/month/year)* | (Earliest) Priority Date *(day/month/year)* |
| PCT/GB 99/04352 | 22/12/1999 | 22/12/1998 |

Applicant

ISIS INNOVATION LIMITED et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

METHOD OF SEQUENCE IDENTIFICATION

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

# INTERNATIONAL SEARCH REPORT

Inter[redacted]al Application No
PCT/GB 99/04352

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JACOBS W R ET AL: "RAPID ASSESSMENT OF DRUG SUSCEPTIBILITIES OF MYCOBACTERIUM TUBERCULOSIS BY MEANS OF LUCIFERASE REPORTER PHAGES" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 260, no. 5109, 7 May 1993 (1993-05-07), pages 819-822, XP000673145 ISSN: 0036-8075 the whole document<br>---<br>-/-- | 1-18 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 18 May 2000 | 29/05/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Molina Galan, E |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DATABASE WPI<br>Week 199522<br>Derwent Publications Ltd., London, GB;<br>AN 170454<br>XP002138099<br>& ZA 9 402 779 A (AMERICAN CYANAMID),<br>26 March 1995 (1995-03-26)<br>abstract | 1-18 |
| X | WO 90 12887 A (BIO ORBIT OY)<br>1 November 1990 (1990-11-01)<br>the whole document | 1-18 |
| X | WO 98 35054 A (RIBOGENE INC)<br>13 August 1998 (1998-08-13)<br>the whole document | 1-18 |
| X | WO 95 19446 A (VIRTA MARKO ;KARP MATTI (FI)) 20 July 1995 (1995-07-20)<br>the whole document | 1-8,<br>14-18 |
| X | WO 97 05241 A (CREATIVE BIOMOLECULES INC)<br>13 February 1997 (1997-02-13)<br>figure 1 | 1-8,<br>14-18 |
| X | DERISI J L ET AL: "EXPLORING THE METABOLIC AND GENETIC CONTROL OF GENE EXPRESSION ON A GENOMIC SCALE"<br>SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,,<br>vol. 278, 24 October 1997 (1997-10-24),<br>pages 680-686, XP000700250<br>ISSN: 0036-8075<br>cited in the application<br>the whole document | 15-17 |
| A | EP 0 837 142 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US))<br>22 April 1998 (1998-04-22) | |
| A | KALABAT D Y ET AL: "Chitobiase, a new reporter enzyme."<br>BIOTECHNIQUES, (1998 DEC) 25 (6) 1030-5.<br>JOURNAL CODE: AN3. ISSN: 0736-6205.,<br>XP002106689<br>United States<br>abstract | 1,2 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DATABASE MEDLINE 'Online!<br>US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US<br>NEUMANN S ET AL: "Discoordinate gene expression of gyrA and gyrB in response to DNA gyrase inhibition in Escherichia coli."<br>XP002106690<br>abstract<br>& JOURNAL OF BASIC MICROBIOLOGY, (1997) 37 (1) 53-69. JOURNAL CODE: JOT. ISSN: 0233-111X.,<br>GERMANY: Germany, Federal Republic of | 1,2 |
| P,X | WO 99 64567 A (DUERRENBERGER FRANZ ;DISCOVERY TECHNOLOGIES LTD (CH); KESSMANN HEL) 16 December 1999 (1999-12-16)<br>the whole document | 1-18 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/04352

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| ZA 9402779 A | 09-01-1995 | NONE | |
| WO 9012887 A | 01-11-1990 | FI 891899 A | 21-10-1990 |
| | | DE 69023642 D | 21-12-1995 |
| | | DE 69023642 T | 17-10-1996 |
| | | EP 0469021 A | 05-02-1992 |
| | | ES 2081981 T | 16-03-1996 |
| WO 9835054 A | 13-08-1998 | AU 6155398 A | 26-08-1998 |
| | | US 5998159 A | 07-12-1999 |
| WO 9519446 A | 20-07-1995 | FI 940225 A | 18-07-1995 |
| | | EP 0689608 A | 03-01-1996 |
| | | FI 954344 A | 15-09-1995 |
| | | US 5776681 A | 07-07-1998 |
| WO 9705241 A | 13-02-1997 | US 5932716 A | 03-08-1999 |
| | | AU 715772 B | 10-02-2000 |
| | | AU 6678696 A | 26-02-1997 |
| | | EP 0842268 A | 20-05-1998 |
| | | JP 11510054 T | 07-09-1999 |
| EP 0837142 A | 22-04-1998 | JP 10191990 A | 28-07-1998 |
| WO 9964567 A | 16-12-1999 | AU 4028199 A | 30-12-1999 |

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# PATENT COOPERATION TREATY

# PCT

REC'D 19 JUN 2001
WIPO PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| N.78329A | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/GB99/04352 | 22/12/1999 | 22/12/1998 |
| International Patent Classification (IPC) or national classification and IPC<br>C12Q1/68 | | |
| Applicant<br>ISIS INNOVATION LIMITED et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 14/07/2000 | 15.06.2001 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office - P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk - Pays Bas<br>Tel. +31 70 340 - 2040 Tx: 31 651 epo nl<br>Fax: +31 70 340 - 3016 | Authorized officer<br>Molina Galan, E<br>Telephone No. +31 70 340 3560 |

Form PCT/IPEA/409 (cover sheet) (January 1994)

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/04352

## I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
**Description, pages:**

| | | | | | |
|---|---|---|---|---|---|
| 1-22 | as originally filed | | | | |

**Claims, No.:**

| | | | | | |
|---|---|---|---|---|---|
| 1-18 | as received on | 10/04/2001 | with letter of | 10/04/2001 | |

**Drawings, sheets:**

| | |
|---|---|
| 1/3-3/3 | as originally filed |

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-4 |
| | No: | Claims | 5-18 |
| Inventive step (IS) | Yes: | Claims | |
| | No: | Claims | 1-18 |
| Industrial applicability (IA) | Yes: | Claims | 1-18 |
| | No: | Claims | |

2. Citations and explanations
**see separate sheet**

## VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

## VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
**see separate sheet**

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
**see separate sheet**

## V. Reasoned statement (Continuation)

2.1 CITATIONS

Reference is made to the following documents:

D1: DATABASE WPI Derwent Publications, AN 170454
& ZA 9 402 779 A (AMERICAN CYANAMID), 26 March 1995
D2: WO 90 12887 A (BIO ORBIT OY) 1 November 1990
D3: WO 98 35054 A (RIBOGENE INC) 13 August 1998
D4: US 4761367 (UNIV NORTH CAROLINA) 2 August 1988
(new citation, copy attached)
D5: WO 99 64567 A (DISCOVERY TECHNOLOGIES LTD) 16 December 1999

2.2 NOVELTY (Art. 33(2) PCT)

2.2.1 D1 discloses a method for identifying modulators of a bacterial essential protein (gyrase inhibitors) by a polynucleotide construct comprising a regulatory sequence functionally coupled to said protein and operably linked to a reporter gene.

2.2.2 D2 discloses a method for identifying modulators of a bacterial essential protein by a polynucleotide construct comprising a regulatory sequence functionally coupled to said protein and operably linked to a reporter gene (cf claims).

2.2.3 D3 discloses a method for identifying modulators of a bacterial essential protein by a polynucleotide construct comprising a regulatory sequence functionally coupled to said protein and operably linked to a reporter gene (cf claims).

2.2.4 The regulatory sequences disclosed in these prior art documents would have been detected by the methods of claims 1 or 2. These methods do not seem to impart any special characteristics to the regulatory sequence further to be affected by a feedback mechanism coupled to a bacterial essential protein.

2.2.5 The inhibitors of claims 13-16 do likewise not seem to posses any particular characteristics conferred by the method of detection further to their ability to modulate bacterial essential proteins. Apart from the modulators apparent from D1-D3 any known antibiotic would fall under the scope of claims 13 to 16. so would pharmaceutical compositions containing them and (trivial) methods using them.

2.2.6 The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of claims 5-18 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

2.3 INVENTIVE STEP (Art. 33(3) PCT)

2.3.1 Document D1 is considered to represent the most relevant state of the art and has been discussed above. The subject-matter of claim 1 differs in that regulatory sequences are used as variable in a method using a construct comprising a regulator linked by a feed back mechanism to an essential protein and a reporter gene operably linked to it.

2.3.2 The problem to be solved by the subject matter of claim 14 may therefore be regarded as how to detect regulatory sequences linked by a feedback mechanism to an essential protein. The solution would be to test potential regulatory sequences in the construct mentioned above by influencing the essential protein.

2.3.3 This solution cannot however be considered as involving an inventive step (Article 33(3) PCT) for the following reason:

2.3.3.1 D4 discloses methods for identifying regulatory sequences in which these are operably linked to a reporter molecule and respond to changes of a protein linked to it (cf claims). The person skilled in the art would apply the teachings of D4 to solve the problem stated above without involvement of inventive skills.

2.3.4 D1 is limited to only one specific essential protein, however, the disclosure would not prevent the person skilled in the art from generalizing the teaching. D4 is

directed to eukaryotic regulatory sequences but the same teachings can be expected to be applicable to bacteria. Furthermore, the meaning of "feedback" has to be taken in its broadest sense and any kind of interaction or relationship with a protein and a regulatory sequence (even at the nucleic acid level) is to be understood under this term.

2.3.5 See section VIII below for further comments on claims 2-4.

2.3.6 The present application does therefore not satisfy the criterion set forth in Article 33(3) PCT and the subject-matter of claims 1-18 does not involve an inventive step (Rule 65(1)(2) PCT).

## VI. Certain documents cited (Continuation)

D5, published on 16.12.99, filed on 4.6.99 and claiming priority from 5.6.99.

Although D5 does not constitute prior art within the meaning of Rule 64.1(b), it seems to disclose the features of claims 5-18 (cf claim19, fig. 1). It might therefore be taken into consideration in the regional phase before the EPO. No check has been made as to wether the priority of this application has been validly claimed.

The priority documents pertaining to the present application were not available at the time of establishing this first written opinion. Hence, it is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. If it later turns out that this is not correct, the above document cited in the international search report could become relevant to assess whether the claimed subject matter satisfy the criteria set forth in Art. 33(1) PCT.

## VII. Certain defects (Continuation)

1 Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in documents D1- D4 is not mentioned in the description, nor are these documents identified therein.

## VIII. Certain Observations (Continuation)

1 The application does not meet the requirements of Article 6 PCT because several claims are not clear for the following reasons:

1.1 It is not clear in claim 2 what relationship exists between the expression of a(ny?) bacterial gene and the regulatory sequence which is to be identified. The IPEA understands and has therefore interpreted that the bacterial genes mentioned in claim 2 are under the control of the said regulatory sequence affected by feedback mechanism coupled to the bacterial essential protein "behaviour". In that case the "bacterial gene" of claim 2 can be considered to be equivalent to the "reporter gene" of claim 1 and both claims have in fact the same scope.

1.2 Claims 13-16 are directed to inhibitors of bacterial essential proteins. However, no such compounds are defined in the application thereby rendering the subject matter of said claims purely speculative and mere statement of the result to be achieved. Moreover, the scope of these claims is open ended and therefore unclear.

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* N.78329A

**Box No. I TITLE OF INVENTION**

ASSAY AND METHOD OF SEQUENCE IDENTIFICATION

**Box No. II APPLICANT**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ISIS INNOVATION LIMITED
British body corporate
of 2 South Parks Road
Oxford OX1 3UB
United Kingdom

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ERRINGTON, Jeffrey
Sir William Dunn School of Pathology
Chemical Pathology Unit
University of Oxford
South Parks Road
Oxford, OX1 3RE

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

**Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ELLIS-JONES, Patrick George Armine
J.A. KEMP & CO.,
14 South Square,
Gray's Inn,
London, WC1R 5LX,
United Kingdom.

Telephone No. +44 171 405 3292

Facsimile No. +44 171 242 8932

Teleprinter No. 23676

☐ **Adress for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No.V DESIGNATION OF STATES**

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

**Regional Patent**

- ☐ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☐ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☐ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

**National Patent** *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☐ **AE** United Arab Emirates
- ☐ **AL** Albania
- ☐ **AM** Armenia
- ☐ **AT** Austria
- ☐ **AU** Australia
- ☐ **AZ** Azerbaijan
- ☐ **BA** Bosnia and Herzegovina
- ☐ **BB** Barbados
- ☐ **BG** Bulgaria
- ☐ **BR** Brazil
- ☐ **BY** Belarus
- ☐ **CA** Canada
- ☐ **CH and LI** Switzerland and Liechtenstein
- ☐ **CN** China
- ☐ **CU** Cuba
- ☐ **CZ** Czech Republic
- ☐ **DE** Germany
- ☐ **DK** Denmark
- ☐ **EE** Estonia
- ☐ **ES** Spain
- ☐ **FI** Finland
- ☐ **GB** United Kingdom
- ☐ **GD** Grenada
- ☐ **GE** Georgia
- ☐ **GH** Ghana
- ☐ **GM** Gambia
- ☐ **HR** Croatia
- ☐ **HU** Hungary
- ☐ **ID** Indonesia
- ☐ **IL** Israel
- ☐ **IN** India
- ☐ **IS** Iceland
- ☒ **JP** Japan
- ☐ **KE** Kenya
- ☐ **KG** Kyrgyzstan
- ☐ **KP** Democratic People's Republic of Korea
- ☐ **KR** Republic of Korea
- ☐ **KZ** Kazakhstan
- ☐ **LC** Saint Lucia
- ☐ **LK** Sri Lanka
- ☐ **LR** Liberia
- ☐ **LS** Lesotho
- ☐ **LT** Lithuania
- ☐ **LU** Luxembourg
- ☐ **LV** Latvia
- ☐ **MD** Republic of Moldova
- ☐ **MG** Madagascar
- ☐ **MK** The former Yugoslav Republic of Macedonia
- ☐ **MA** Morocco
- ☐ **MN** Mongolia
- ☐ **MW** Malawi
- ☐ **MX** Mexico
- ☐ **NO** Norway
- ☐ **NZ** New Zealand
- ☐ **PL** Poland
- ☐ **PT** Portugal
- ☐ **RO** Romania
- ☐ **RU** Russian Federation
- ☐ **SD** Sudan
- ☐ **SE** Sweden
- ☐ **SG** Singapore
- ☐ **SI** Slovenia
- ☐ **SK** Slovakia
- ☐ **SL** Sierra Leone
- ☐ **TJ** Tajikistan
- ☐ **TM** Turkmenistan
- ☐ **TR** Turkey
- ☐ **TT** Trinidad and Tobago
- ☐ **UA** Ukraine
- ☐ **UG** Uganda
- ☒ **US** United States of America
- ☐ **TZ** Tanzania
- ☐ **UZ** Uzbekistan
- ☐ **VN** Viet Nam
- ☐ **YU** Yugoslavia
- ☐ **ZA** South Africa
- ☐ **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☐ **CR** Costa Rica
- ☐ **DM** Dominica

**Precautionary Designation Statement:** In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn bythe applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

**Box No. VI PRIORITY CLAIM** ☐ Further priority claims are indicated in the Supplemental Box.

| Filing date of earlier application (day/month/year) | Number of earlier application | Where earlier application is: national application: country | regional application:* regional Office | international application: receiving Office |
|---|---|---|---|---|
| item (1) 22.12.98 (22 Dec 1998) | 98310567.7 | | EPO | |
| item (2) | | | | |
| item (3) | | | | |

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

**Box No. VII INTERNATIONAL SEARCHING AUTHORITY**

**Choice of International Searching Authority (ISA)** *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

**Request to use results of earlier search; reference to that search** *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

**Box No. VIII CHECK LIST; LANGUAGE OF FILING**

This international application contains the following **number of sheets:**

| | |
|---|---|
| request | : 3 |
| description (excluding sequence listing part) | : 22 |
| claims | : 3 |
| abstract | : 1 |
| drawings | : 3 |
| sequence listing part of description | : |
| **Total number of sheets** | : 32 |

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

**Figure of the drawings** which should accompany the abstract:

**Language of filing** of the international application:

**Box No. IX SIGNATURE OF APPLICANT OR AGENT**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).*

ELLIS-JONES, Patrick George Armine

**For receiving Office use only**

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

**For International Bureau use only**

Date of receipt of the record copy by the International Bureau:

*This sheet is not part of and does not count as a sheet of the international application.*

# PCT

## FEE CALCULATION SHEET

**Annex to the Request**

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference N.78329A

Applicant ISIS INNOVATION LTD

### CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £ 55 [T]
2. SEARCH FEE . . . . . £ 638 [S]

International search to be carried out by _____
*(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)*

3. INTERNATIONAL FEE

**Basic Fee**

The international application contains 32 sheets.

first 30 sheets . . . . . £ 285 [b1]

2 (remaining sheets) x £6 (additional amount) = £ 12 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . £ 297 [B]

**Designation Fees**

The international application contains 3 designations.

3 (number of designation fees payable *(maximum 10)*) x £65 (amount of designation fee) = £ 195 [D]

Add amounts entered at B and D and enter total at I . . . . . £ 492 [I]

*(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)*

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . £ [P]

5. TOTAL FEES PAYABLE . . . . . £ 1185 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

### MODE OF PAYMENT

- ☐ authorization to charge deposit account (see below)
- ☒ cheque
- ☐ postal money order
- ☐ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify)*:

### DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____

- ☐ is hereby authorized to charge the total fees indicated above to my deposit account.
- ☐ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.
- ☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No. _____ Date *(day/month/year)* _____ Signature _____

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

# PCT

## NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

ELLIS-JONES, Patrick, George, Armine
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

J. A. KEMP & Co
- 7 APR 2000
Action by PEOTSER

| | |
|---|---|
| Date of mailing (day/month/year)<br>28 March 2000 (28.03.00) | |
| Applicant's or agent's file reference<br>N.78329A | **IMPORTANT NOTIFICATION** |
| International application No.<br>PCT/GB99/04352 | International filing date (day/month/year)<br>22 December 1999 (22.12.99) |
| International publication date (day/month/year)<br>Not yet published | Priority date (day/month/year)<br>22 December 1998 (22.12.98) |
| Applicant<br>ISIS INNOVATION LIMITED et al | |

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

| Priority date | Priority application No. | Country or regional Office or PCT receiving Office | Date of receipt of priority document |
|---|---|---|---|
| 22 Dece 1998 (22.12.98) | 98310567.7 | EP | 23 Marc 2000 (23.03.00) |

**The International Bureau of WIPO**
**34, chemin des Colombettes**
**1211 Geneva 20, Switzerland**

Facsimile No. (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:
ELLIS-JONES, Patrick, George, Armine
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

J. A. KEMP & Co
REC'D - 7 JUL 2000
Action by SGL

# NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>29 June 2000 (29.06.00) | |
| **Applicant's or agent's file reference**<br>N.78329A ✓ | **IMPORTANT NOTICE** |
| **International application No.**<br>PCT/GB99/04352 ✓ | **International filing date** (day/month/year)<br>22 December 1999 (22.12.99) ✓ |
| **Priority date** (day/month/year)<br>22 December 1998 (22.12.98) | |
| **Applicant**<br>ISIS INNOVATION LIMITED et al ✓ | |

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

   JP,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

   EP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on

   29 June 2000 (29.06.00) under No. WO 00/37676

## REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

## REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

| | |
|---|---|
| **The International Bureau of WIPO**<br>**34, chemin des Colombettes**<br>**1211 Geneva 20, Switzerland** | Authorized officer<br>J. Zahra |
| Facsimile No. (41-22) 740.14.35 | Telephone No. (41-22) 338.83.38 |

Form PCT/IB/308 (July 1996)

3367268

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

| Date of mailing (day/month/year) | IMPORTANT NOTICE |
|---|---|
| 29 June 2000 (29.06.00) | |
| **Applicant's or agent's file reference** | **International application No.** |
| N.78329A | PCT/GB99/04352 |

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

*The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The name or two-letter code of that Authority may be indicated by the applicant on the line below:*

IPEA/ EP

# PCT

**CHAPTER II**

## DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

| Identification of IPEA | Date of receipt of DEMAND |
|---|---|
| | |

**Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION**

Applicant's or agent's file reference: N.78329A

| International application No. | International filing date *(day/month/year)* | (Earliest) Priority date *(day/month/year)* |
|---|---|---|
| PCT/GB99/04352 | 22 December 1999 | 22 December 1998 |

Title of invention: METHOD OF SEQUENCE IDENTIFICATION

**Box No. II APPLICANT(S)**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ISIS INNOVATION LIMITED
2 South Parks Road
Oxford OX1 3UB
United Kingdom

Telephone No.:

Facsimile No.:

Teleprinter No.:

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| GB | GB |

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ERRINGTON, Jeffrey
Sir William Dunn School of Pathology
Chemical Pathology Unit
University of Oxford
South Parks Road
Oxford, OX1 3RE

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| GB | GB |

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

| State *(that is, country)* of nationality: | State *(that is, country)* of residence: |
|---|---|
| | |

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/GB99/04352

**Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The following person is [x] agent [ ] common representative

and [x] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ELLIS-JONES, Patrick George Armine
J.A. KEMP & CO.,
14 South Square,
Gray's Inn,
London, WC1R 5LX,
United Kingdom.

Telephone No.: +44 20 7405 3292

Facsimile No.: +44 20 7242 8932

Teleprinter No.: 23676

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION**

**Statement concerning amendments:** *

1. The applicant wishes the international preliminary examination to start on the basis of:

[x] the international application as originally filed

the description
[ ] as originally filed
[ ] as amended under Article 34

the claims
[ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings
[ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

**Language for the purposes of international preliminary examination:** English

[x] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

**Box No. V ELECTION OF STATES**

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**



*See Notes to the demand form*

ational application No.
PCT/GB99/04352

**Box No. VI CHECK LIST**

| The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination: | | | For International Preliminary Examining Authority use only: received | For International Preliminary Examining Authority use only: not received |
|---|---|---|---|---|
| 1. translation of international application | : | sheets | ☐ | ☐ |
| 2. amendments under Article 34 | : | sheets | ☐ | ☐ |
| 3. copy (or, where required, translation) of amendments under Article 19 | : | sheets | ☐ | ☐ |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets | ☐ | ☐ |
| 5. letter | : 1 | sheets | ☐ | ☐ |
| 6. other *(specify)* | : | sheets | ☐ | ☐ |

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

**Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).*

ELLIS-JONES, Patrick George Armine
AUTHORISED REPRESENTATIVE

DATE: 11 July 2000

**For International Preliminary Examining Authority use only**

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

**For International Bureau use only**

Demand received from IPEA on:



*See Notes to the demand form*

PATENT COOPERATION TREAT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

# PCT

To:
ELLIS-JONES, Patrick G.
J.A. KEMP & CO
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

J. A. KEMP & Co
26 JUL 2000
Action by

## NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*: 24.07.00

Applicant's or agent's file reference: N.78329A

**IMPORTANT NOTIFICATION**

| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
|---|---|---|
| PCT/GB 99/04352 | 22/12/1999 | 22/12/1998 |

Applicant: ISIS INNOVATION LIMITED et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

14/07/2000

2. This date of receipt is:

☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).

☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).

☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

☐ *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Netherlands
Tel.: (+31-70) 340-2040, Tx. 31 651 epo nl
Fax: (+31-70) 340-3016

Authorized officer
KRUYDENBERG G L M
Tel. (+31-70) 340-2277

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

ELLIS-JONES, Patrick, George,
Armine
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

J. A. K
16
Action by

## INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>10 August 2000 (10.08.00) | |
| **Applicant's or agent's file reference**<br>N.78329A | **IMPORTANT INFORMATION** |

| **International application No.** | **International filing date** (day/month/year) | **Priority date** (day/month/year) |
|---|---|---|
| PCT/GB99/04352 | 22 December 1999 (22.12.99) | 22 December 1998 (22.12.98) |

**Applicant**
ISIS INNOVATION LIMITED et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :JP,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   None

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

**The International Bureau of WIPO**
**34, chemin des Colombettes**
**1211 Geneva 20, Switzerland**

Facsimile No. (41-22) 740.14.35

Authorized officer:
Pascal Piriou

Telephone No. (41-22) 338.83.38

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68

A1

(11) International Publication Number: **WO 00/37676**

(43) International Publication Date: 29 June 2000 (29.06.00)

(21) International Application Number: PCT/GB99/04352

(22) International Filing Date: 22 December 1999 (22.12.99)

(30) Priority Data:
98310567.7 22 December 1998 (22.12.98) EP

(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: ERRINGTON, Jeffrey [GB/GB]; Sir William Dunn School of Pathology, Chemical Pathology Unit, University of Oxford, South Parks Road, Oxford OX1 3RE (GB).

(74) Agent: ELLIS–JONES, Patrick, George, Armine; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: METHOD OF SEQUENCE IDENTIFICATION

(57) Abstract

Cells of *Bacillus subtilis* or other micro–organism suitable for screening compounds for antibiotic activity express an essential protein and have a polynucleotide construct comprising a regulatory sequence operably linked to a reporter gene, in which the regulatory sequence is associated with a feedback mechanism responsive to alteration of synthesis or activity of the essential protein. A method of screening compounds for antibiotic or other biological activity comprises incubating the compounds with aliquots of the cells as defined and observing the level of expression of the reporter gene. Methods of identifying suitable regulatory sequences which are not promoters of the target gene, are also described.

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# METHOD OF SEQUENCE IDENTIFICATION

## Introduction

There is an urgent need to identify and develop new families of antibiotics to combat the continuing threat of antibiotic resistance. Most major pharmaceutical companies are using the following general approach to identify new antibiotics.

1. Target identification. Genes that appear to be essential for bacterial viability are first identified. Most often, this is based on inviability of the cells containing a form of the gene that has been inactivated. Information from genomic DNA sequence is used to identify genes that are conserved in bacteria but not in humans. The targeting of such genes is thought to be more likely to provide inhibitors that will be specific to bacterial cells - a crucial feature for efficacy as an antibiotic.

2. Assay development. A suitable means of screening for inhibition of the function of the target gene is then devised. This can be either an *in vitro*, biochemical assay, or a whole cell assay.

3. High throughput screening. The assay is configured in such a way that a very large number of chemical compounds can be screened for activity against the target function. Many pharmaceutical companies have access to very large collections of compounds that can be screened. Compounds detected in this way provide lead molecules from which antibodies can potentially be derived.

Most pharmaceutical companies now have good resources with which to underpin activities 1 and 3. However, there is a major bottleneck in the development of assays, especially for the large number of genes of unknown function that are potentially good targets.

Most important functions in living cells, especially bacteria and lower eukaryotes, are tightly regulated. Feedback mechanisms often ensure that products are made only in amounts sufficient to fulfil their functions. There are numerous examples of feedback regulation at the level of transcription or translation; for example, transcription attenuation to control tRNA synthetase (Henkin, 1994) and ribonucleotide precursor synthesis (Lu *et al*, 1996); DNA supercoiling controls the promoter for DNA gyrase (Menzel & Gellert, 1983). The same is likely to occur at

the promoters of many other genes, including genes for cell division, DNA replication, etc.

The present invention makes use of this feedback regulation, hereinafter sometimes called autoregulation, to provide whole cell assays for screening compounds for antibiotic or other biological activity. It may be convenient for this purpose to make use (to effect expression of a reporter gene) of a promoter for the target gene. Alternatively other regulatory sequences, e.g. promoters of other genes of known or unknown function, may be used for the purpose. It is another object of this invention to provide a means of identifying such regulatory sequences.

**The Invention**

In a first aspect, the invention provides a method for identifying a modulator of a bacterial essential protein comprising:

i) providing a bacterial host cell which expresses the essential protein and having a polynucleotide construct comprising a regulatory sequence operably linked to a reporter gene wherein the regulatory sequence is associated with a feed back mechanism responsive to changes in the synthesis or activity of the essential protein;

ii) contacting a test substance with the host cell; and

iii) monitoring expression of the reporter gene to determine thereby whether the said substance modulates the synthesis or activity of the essential protein.

In another aspect, the invention provides an inhibitor of a bacterial essential protein identifiable by the method of the invention; an inhibitor of a bacterial essential protein identified according to the method of the invention; an inhibitor as defined above for use in a method of treatment of a human or animal body; or an inhibitor as defined above for use as an antibiotic.

The invention also provides a pharmaceutical composition comprising the inhibitor as defined above and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method for identifying a

regulatory sequence which is affected by a feedback mechanism on alteration of synthesis or activity of a bacterial essential protein comprising:

i) providing a bacterial cell having a reporter gene under the control of a candidate regulatory sequence;

ii) selecting a target essential protein which is expressed in the organism;

iii) altering the synthesis or activity of the essential protein; and

iv) monitoring expression of the reporter gene.

v) determining thereby whether the candidate regulatory sequence is affected by a feedback mechanism.

The invention also provides a method of identifying a regulatory sequence whose activity is affected by a feedback mechanism on alteration of the synthesis or activity of an essential bacterial protein comprising monitoring gene expression in the presence of normal and altered synthesis or activity of the essential protein, and identifying thereby a regulatory sequence whose activity is affected by a feedback mechanism associated with the essential bacterial protein.

In another aspect the invention provides cells of an organism suitable for screening compounds for biological activity, which cells contain a chromosome including:

a) a target gene whose expression or activity is subject to a feedback mechanism, and

b) an artificially introduced reporter gene under the control of a regulatory sequence associated with the said feedback mechanism, whereby a reduction of synthesis or activity of a target gene expression product is associated with an increase in the expression of the reporter gene.

In another aspect the invention provides a method of screening compounds for biological activity, which method comprises incubating the compounds with aliquots of the cells as defined, and observing the level of expression of the reporter gene.

In another aspect, the invention provides compounds e.g. antibiotics identified by the method; and use of the compounds so identified to treat, e.g. kill or inhibit the growth of, bacteria.

The invention provides a method for screening for an inhibitor or modulator of a bacterial essential protein. The essential protein is encoded by a target gene and may be of known or unknown function. Alteration of expression or activity of the target essential protein leads to a feedback mechanism in the bacteria, which serves to alter or up regulate expression from a promoter or regulatory sequence of a bacterial gene.

The nature of the feedback mechanism is not critical; it may operate at the RNA level or at the protein level and may involve transcription and/or translation of the target gene and/or activity of a target gene expression product. The target gene will generally encode a protein that is essential for viability and well conserved in bacteria or other micro-organisms but absent from or highly dissimilar in higher organisms e.g. mammals or humans.

When expression of the target gene or synthesis or activity of a target gene expression product is altered (the means by which that alteration is effected being unimportant for present purposes), then a feedback mechanism may operate in the bacterial cell to compensate for the alteration. The feedback mechanism may operate on a regulatory sequence e.g. a promoter of the target gene. Thus, for example a reduction of synthesis or activity of a target gene expression product may be associated, via the feedback mechanism, with an increase in activity of the promoter of the target gene. In accordance with an assay of the invention, a polynucleotide construct is provided in the bacterial cell comprising a reporter gene under the control of the promoter of the target gene.

Alternatively or additionally, a reduction in the synthesis or activity of the essential protein may be associated with an alteration, and generally an increase in the expression of another bacterial gene, for example, through up regulation of the promoter for that gene. In an alternative embodiment of the invention, a polynucleotide construct is provided comprising a reporter gene under the control of the promoter or other regulatory sequence that is not associated with the target essential protein *per se* but is subject to upregulation through a feed back mechanism when the activity or synthesis of the essential protein is altered.

In some instances, it may not be possible to predict which genes will be

involved in a feed back mechanism other than a promoter of the target gene. The invention also provides methods of identifying potential genes which are subject to a feed back mechanism effected by alterations in the activity or synthesis of the essential functional protein under investigation. Once such a gene has been identified, a reporter gene under the control of the promoter or regulatory sequence for that gene may be readily constructed and incorporated in a bacterial cell for use in accordance with an assay of the invention.

The cells of the invention thus contain an artificially introduced reporter gene under the control of a regulatory sequence associated with the target gene that is to say affected by a feedback mechanism associated with a reduction in the synthesis or activity of the target gene product. In an alternative aspect of the invention the reporter gene comprises the gene whose expression is altered by the feedback mechanism described above and the assay comprises monitoring for up regulation for that gene, for example using a gene array or proteomics technique to monitor transcription or expression of the gene.

The target gene in accordance with the present invention encodes a protein which is essential for bacterial viability such that a test substance which effects the essential protein, either effecting its synthesis or activity may be useful as an antibiotic. As highlighted above, in accordance with the present invention, such a target gene must also in some way be associated with a feedback mechanism effecting either the expression of the target gene itself or other genes within the bacterial cell. The promoters or other regulatory sequences of the target gene or genes affected by the feedback mechanism may thus be useful when coupled to a reporter gene in an assay. By monitoring for expression of the reporter gene, a substance which inhibit synthesis or activity of the target protein may be identified. Proteins involved in cell wall precursor synthesis, teichoic acid synthesis, DNA replication, RNA synthesis, cell division, chromosome segregation, translation and other essential genes are all suitable targets for use in accordance with the invention. Some specific examples are discussed in more detail below, by way of example only.

The following are envisaged as preferred target genes for use in *B. subtilis* or other bacteria. These may include proteins involved in the following

process:

Cell wall (peptidoglycan) precursor synthesis (including the products of the genes: *murA,B,C,D,E,F,G,Z, dal, dap, ddlA*);

Teichoic acid synthesis (*tagA,B,C,D,E,F,G,H, gtaB*);

DNA replication (*dnaA, gyrA,B, topA,B, lig[yerG]*);

RNA synthesis (*sigA, rpoA, rpoB, rpoC*);

Cell division (*ftsA,L,W,Z, divIB, divIC, divIVA, pbpB);*

Chromosome segregation (*spoIIIE, soj, spoOJ codV, ripX*);

Translation (*infA, B, C, fmt, efp);*

Miscellaneous (*obg, lgt);*

Essential genes of unknown function discovered by the inventor (*yjbN, yloQ*).

Lipid synthesis *(pgsA)*.

Examples of target genes are discussed in more detail below.

**DnaA - an example of a DNA replication protein**

The *dnaA* gene is widely conserved among bacterial species and in *E.coli* and *B. subtilis* at least, it is essential, being required for initiation of DNA replication (although the Cyanobacterial gene was recently reported to be non-essential; Richter *et al.*, 1998, *J. Bacteriol.* **180**, 4946-4949). Eukaryotes, in contrast, use a quite different mechanism to initiate chromosome replication, so agents that specifically inhibit DnaA function should have selective toxicity against bacteria. Accumulation of DnaA during the cell cycle is thought to result in gradual filling of binding sites ("DnaA boxes") in the *oriC* region. Eventually, enough DnaA accumulates to allow formation of an initiation complex that leads to the initiation of bidirectional chromosome replication. In *B. subtilis* at least, the *dnaA* gene is monocistronic and located right next to *oriC* (Moriya *et al.*, 1988, EMBO J. 7, 2911-2917). There is a long upstream regulatory region containing several DnaA boxes. Two of these appear to overlap the promoter region of the gene. There is well-documented evidence in both *E.coli* and *B. subtilis* that DnaA negatively autoregulates, so depletion of DnaA is compensated for by increased transcription from its promoter. Preliminary experiments are in progress to show that depletion of DnaA protein (by

artificially repressing its gene) result in increased transcription from the *dnaA* promoter. If this works, reporter genes coupled to the *dnaA* promoter should provide a convenient means of screening for inhibitors of this excellent antibiotic target. Promoters to genes uninvolved with DNA replication (e.g. the $P_{xyl}$ promoter, induced by the presence of xylose) should be either unaffected by inhibitors of DnaA, or else gradually shut down, as the cells begin to die through loss of their DNA. It is possible that the arrest in initiation of DNA replication caused by depletion or inhibition of DnaA will result in repression of genes involved in other steps in DNA replication. The promoters of such genes might provide better control promoters to ensure the specificity of inhibitors against DnaA, rather against DNA replication in general. A dual reporter system based on the $P_{dnaA}$ and possibly $P_{xyl}$ promoters should provide a sensitive and specific whole cell assay for inhibitors of DnaA function.

**Dal - an example of a protein involved in a cell wall synthesis**

Bacterial cell walls are essential stress-bearing structures made of peptidoglycan (PG). A wall is present in virtually all eubacteria. It is composed of long glycan (amino sugar) polymers crosslinked by short peptides. The peptides are unusual in that they contain amino acids in the D-isomeric state. There is no structure equivalent to the cell wall in higher eukaryotes and D-amino acids are generally absent. Many important antibiotics, including penicillins, cephalosporins and vancomycin act on PG synthesis. D-alanine, which is universally found in PG, is obtained by bacteria either from their growth medium or by the action of an enzyme, D-alanine racemase, encoded by the *dal* gene, which converts L-alanine to the D-form. *dal* is a very highly conserved gene in eubacteria but absent from eukaryotes.

Tests are in progress to determine whether the *dal* promoter is autoregulated; its promoter is expected to be up-regulated in response to depletion of the Dal protein. If this is the case, the promoter can be used to screen for inhibitors of Dal. $P_{xyl}$ can again be used as a control, although there might be better promoters (e.g. one responding negatively to increases in the substrate used by Dal).

**InfC - an example of a protein involved in translation**

The process of translation initiation is highly conserved in bacteria but differs fundamentally from eukaryotes. Thus, initiation codons are defined by a "ribosome-binding site" (RBS), which lies close to the initiation codon, which is usually AUG but sometimes UUG or GUG.

In humans, the first AUG in the mRNA tends to be used, irrespective of the context - there is no equivalent of the RBS. Translation initiation factor 3 of bacteria is highly conserved in bacteria and seems to be required for specificity of initiation at start codons. In both *B. subtilis* and *E.coli*, the initiator codon of the *infC* gene is highly unusual - AUU. Probably, this codon can only be used to initiate translation and thus make more InfC protein, when the factor is limiting, allowing the ribosome to initiate less stringently. Experiments with *E.coli* support this (reviewed by Grunberg-Manago, 1996, In *Escherichia coli* and *Salmonella typhimurium*: *cellular and molecular biology*, eds Neidhardt *et al.*, ASM Press, Washington D.C. pp 1432-1457). A highly specific whole assay for inhibitors of InfC is envisaged based on a use of a pair of reporter genes. Both will carry a promoter driving transcription of the *infC* translation initiation region; in one case, the reporter gene will be fused in frame with the beginning of the *infC* coding region, including the AUU initiation codon; the other will have a different reporter gene fused to an identical leader except with AUG as initiator. The first reporter should induce when InfC is depleted or inhibited. The latter will provide a control essentially unresponsive to changes in the levels of InfC.

**Fmt - an example of a protein involved in the translation**

Methionyl-tRNA formyltransferase (Fmt) carries out the final step in synthesis of the initiator tRNA in bacteria. Bacterial cells use a special initiator tRNA, $tRNA_{Fmt}$, specifically at start codons. Eukaryotic cells use the normal tRNA for methionine at both initiator and internal codons and so have no need of Fmt. Disruption of the *fmt* gene of *E.coli* (at least) causes a severe growth defect (Guillon, J.-M., Mechulam, Y., Schmitter, J.-M., Blanquet, S., Fayat, G. (1992) J. Bacteriol, **174**, 4294-4301), so its product is likely to be a good target for antibiotics. In the gene sequence, there is an unusual RBS with an ATG right next to it, followed by an

Ile codon and a stop. The correct initiator AUG follows. In the presence of excess Fmt, binding is expected to occur at the inappropriate upstream AUG and this might block use of the correct start codon. This could be tested with wild type and mutant initiator regions fused in frame to *lacZ*, as for *infC*. It is expected that these reporters will respond appropriately to depletion of Fmt, and will provide a specific assay for inhibitors of Fmt.

In one aspect of the invention a second polynucleotide construct is provided in the bacterial host cell comprising a second reporter gene linked to a second promoter. The second promoter is provided such that under the conditions of the assay, in the absence of a test substance the second reporter gene would normally be expected to be expressed. This allows for non specific effects of the test substance to be identified. For example, if the test substance is a general inhibitor of translation within the bacterial cell, no expression of the second reporter gene would be expected.

The first and/or second reporter genes encode products which can readily be detected. For example, the reporter product may be detected by fluorescent, luminescent or other standard reporting techniques. The reporter gene products may comprise an enzyme such as β-galactosidase, production of which may be identified by use of colorogenic or fluorogenic enzymes substrates. Other reporter genes include β-glucuronidase, green fluorescent protein (GFP) and variants thereof, luciferase, chloramphenicol acetyl transferase, catechol oxidase, an antigen which may readily be recognised by an antibody, other affinity ligands such as streptavadin/biotin or protein A which may be detected by antibodies etc. The first and second reporter genes where present are selected such that it is possible to differentiate between expression of the first reporter gene and expression of the second reporter gene. In an alternative aspect of the invention, no second reporter gene is provided. However, other properties such as the optical density of the bacterial culture may be monitored with a view to assessing to what extent non-specific effects are occurring based on changes in culture growth which may be taken into account when assessing the effect of the test substance.

The polynucleotide construct(s) comprise a promoter operably linked to the reporter gene to be expressed. Preferably, where a second polynucleotide construct is provided, the second promoter comprises an inducible promoter. The conditions required to induce this promoter can be applied to the host cell when adding the test substance during the course of the assay. Such inducing conditions would allow for the expression of the second reporter gene in the absence of the test substance. Examples of inducers include xylose, tetracycline, lactose and derivatives thereof including IPTG, arabinose and gluconate or a change of temperature. For example, if the promoter is controlled by a temperature-sensitive repressor, the promoter can be induced by increased temperatures.

The regulatory sequence or promoters associated with the feedback mechanism may comprise an endogenous bacterial promoter, or a polynucleotide homologous to such an endogenous promoter which retains the activity of the promoter i.e. is affected by the feedback mechanism. A non-naturally occurring promoter may be provided comprising responsive elements which respond to the feedback mechanism coupled to other regulatory sequences sufficient to operably express the reporter gene via the feedback mechanism.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

The polynucleotide construct(s) may be provided as vector(s) for transformation of the bacterial host cell. The polynucleotide constructs may be provided on the same or different vectors. Vectors may be used to replicate the vectors in a compatible host cell. The vectors may be for example plasmid vectors provided with an origin of replication and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes such as ampicillin or chloramphenicol resistance genes for selection in bacterial cells.

In an alternative aspect, the invention also relates to a host cell transformed, conjugated or transduced with first and second polynucleotide constructs for the

expression of the first and second reporter genes.

The assay of the invention is used to screen for compounds which modulate synthesis or activity of the bacterial essential protein. Any suitable format may be used for the assay for identifying a modulator of bacterial protein activity (the target protein). The way in which the assay is carried out will depend in part of the nature of the first and where present second reporter genes. In some instances it may be necessary to divide a sample containing the host cells following administration of the test substance in order to monitor separately for first and second reporter gene activity. The host cells are preferably bacterial cells and may be selected from *Bacillus subtilis, E coli, Salmonella, Streptococcus, Staphylococcus etc.* The conditions of the assay are selected such that the host bacterial cell may grow in the absence of the test substance. Preferably the assay is carried out under conditions which allow for the feedback mechanism to operate if there is an alteration of the synthesis or activity of the target protein such that the reporter gene is expressed.

Additional control experiments may be appropriate. The progress of the assay can be followed in the presence and in the absence of the test substance. Known target protein modulators, may be used as positive controls in order to show a comparable or similar effect in a test substance. Known antibiotics may be tested to demonstrate their effect on the target protein. Additional assays may be carried out for example on mammalian cells or in a mammalian host to check that the test substance does not show adverse side effect on such host cells.

Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display (e.g. phage display libraries) and antibody products.

Test substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition or activation tested individually. Test substances may be used at concentrations from 1μM to 10mM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM. Complex mixtures of natural origin (e.g. filtrates from bacterial cultures, or plant extracts) may be used.

Inhibitors of target bacterial protein activity may be used to restrict the growth of bacteria. Such inhibitors may be used to treat bacterial conditions in humans or animals and thus may be used as antibiotics to treat such bacterial infection.

Such inhibitors may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The inhibitors may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The modulators may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a modulator for use in prophylaxis or treatment will depend upon factors such as the nature of the exact modulator, whether a pharmaceutical or veterinary use is intended, etc. A modulator may be formulated for simultaneous, separate or sequential use.

A modulator of target protein activity is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or

saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a modulator is administered to a patient. The dose of modulator may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg, preferably from about 0.1mg/kg to 10mg/kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the infection and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

A high throughput screen runs in the following way. A strain of *B. subtilis* is constructed containing two reporter genes encoding enzymes or proteins that can be detected, such as β-galactosidase and β-glucuronidase. Reporter genes capable of being expressed in *Bacillus* species are well known and documented in the literature. Reporter genes are preferably chosen so that their products can be readily assayed simultaneously. Green fluorescent protein has the advantage that its intrinsic fluorescence allows the protein to be assayed by direct fluorimetric measurement. *LacZ* has been used for more than 10 years with great success in *B. subtilis* and there is a range of useful substrates that generate coloured or fluorescent products upon hydrolysis by β-galactosidase. The *uidA* gene of *E.coli* has recently been harnessed for similar purposes, and the range of substrates available for the gene product, β-

glucuronidase is similar to that for β-galactosidase. Two different fluorogenic substrates may be used to assay the activities of the two reporters simultaneously in a single reaction. One reporter is fused to a sequence that causes increased expression in the absence of the desired function (e.g. gyrase); the other to a sequence that gives unchanged or decreased expression. The dual reporter strain is grown in an appropriate medium, dispensed into a vessel allowing large scale screening, such as the wells of microtitre plates. Each well would contain at least one test compound. The cells would be incubated for an appropriate time period (preferably two or more cell doublings), after which (if necessary, depending on the reporters used) a reaction cocktail allowing simultaneous assay of the two reporter enzymes would be added. The assays might be based on enzyme substrates giving e.g. chemiluminescent or fluorescent or coloured products. For fluorogenic substrates the presence of either or both enzymes may be detected simultaneously by a fluorimeter set to receive two different appropriate wavelengths. In the example described above, a positive response, in terms of inhibition of DNA gyrase, would be indicated by increased activity of β-galactosidase and decreased β-glucuronidase. Compounds eliciting such a response would be potential antimicrobial agents with DNA gyrase as the likely target.

Alternatively, the chemicals could be individually spotted onto a lawn of cell plated on a growth medium containing colorigenic or fluorogenic substrates. Chemicals eliciting an appropriate response would be detectable by their enhancement or inhibition of enzyme activity in the vicinity of the spot.

In principle, there are many essential genes in bacteria that are likely to be sufficiently different from mammalian cells to provide the selective toxicity needed for a useful antibiotic. Moreover, it is well known that mutations affecting central metabolic functions, such as nucleotide precursor synthesis, lead to attenuation of virulence, because they perturb the ability of the pathogen to grow in host cells or tissues. So this approach may provide not only antibiotics but also drugs that attenuate virulence and which will act synergistically with antibiotics. The strategy is especially important because it could be applied to any gene of interest, even though the precise function of the gene is not known.

The present invention also relates to the use of regulatory sequences or promoters which are not necessarily the promoter of the target gene itself but a promoter which is up-regulated in response to a decrease in the synthesis or activity of the target gene. The invention also provides a method for identifying such a regulatory sequence by looking for differential expression of a gene under the control of a regulatory sequence when the synthesis or activity of an essential protein is altered. In addition, an assay of the invention may involve monitoring differential expression of the gene affected by the feedback mechanism.

Identification of differential expression of a gene under control of the regulatory sequence may be carried out in a number of different ways. In a first embodiment, a library is prepared comprising fragments of the chromosome of a bacterial gene, each fused to a reporter gene. Such constructs are used to transform bacteria. The synthesis or activity of the essential target gene is then altered and expression of the reporter gene is monitored. Those constructs which show upregulation of the reporter gene are then further analysed to identify those regulatory sequences which are upregulated in response to an alteration of the synthesis or activity of the essential target gene. In an alternative embodiment, differential expression maybe monitored using a gene array of nucleotide sequences from the genes of the organism and analysing which genes are upregulated in response to alterations in the synthesis or activity of the target protein. Alternately, such differential expression could be analysed by recovering proteins from the organism and separating the recovered proteins to observe changes resulting from alterations in the synthesis or activity of the target gene. Promoter or regulatory sequences associated with such genes or proteins whose expression is altered in response to alterations in the synthesis or activity of the essential target gene may then be used in accordance with an assay of the invention.

In an assay of the invention, a gene whose expression if affected by alternations in the expression or activity of the target essential protein is selected. Bacterial cells are incubated in the presence and absence of a test substance and differential expression of the gene is investigated by one of the methods outlined above.

When the regulatory sequence is not a promoter of the target gene, then the invention also provides a method of identifying a regulatory sequence, which method comprises the steps of:-

i) selecting a target gene in a chromosome of an organism wherein expression of the target gene is subject to a feedback mechanism,

ii) altering the synthesis or activity of an expression product of the target gene, and

iii) observing a corresponding change in activity of a regulatory sequence associated with the said feedback mechanism,

wherein there is introduced into the chromosome of the organism a reporter gene under the control of the regulatory sequence, and step iii) is performed by observing a corresponding change in the expression of the reporter gene.

Step ii) of that method preferably involves controlling the expression of the target gene through the use of a genetic construct containing a known repressor or promoter sequence so as to modulate a level of an expression product via exposure of the cells to an external chemical or physical inducing factor; or isolating a conditional mutation in the gene, which allows activity of its product only under certain conditions, such as low or high temperature.

In one method there is introduced into the host organism a reporter gene under the control of the regulatory sequence, and step iii) is performed by observing a corresponding change in the expression of the reporter gene. Preferably there is provided a library of constructs of potential regulatory sequences associated with the said feedback mechanism, each potential regulatory sequence fused to a reporter gene. Preferably iii) is performed by genetically modifying aliquots of cells of the organism by introducing into the chromosome of the cells of each aliquot a different construct of the library. The genetic modification may simply comprise introduction of a plasmid containing a construct. The potential regulatory sequences are preferably DNA fragments of approximately 50-1000 bp of the genome of the organism. A library would preferably contain at least 10 different constructs. Preferably the DNA fragments of the library comprise the entire genome of the organism.

Another method involves providing an array of DNA sequences of genes of the organism and monitoring for differential mRNA expression in the presence of functional inhibition of the target gene. Preferably the array is of DNA sequences taken from each of the genes of the organism and provided in spots at spaced locations on a surface of a support. Cells of the organism are incubated under conditions which permit expression of the target gene, RNA is recovered and if desired converted to cDNA. Either the RNA or the cDNA is applied to the array under hybridisation conditions, and a hybridisation pattern is noted. Another aliquot of cells of the organism is incubated under conditions to alter or prevent expression of the target gene. RNA is recovered and RNA or cDNA is applied to the array under hybridisation conditions in order to generate a hybridisation pattern different from the previous one. The differences are noted and are indicative of genes and their regulatory sequences which are involved in a feedback mechanism associated with the target gene. Such gene expression arrays have been described in the literature and used to investigate feedback mechanisms of micro-organisms, but not, it is believed, for the purpose of identifying regulatory sequences for use in screening for antibiotics. (DeRisi, J L *et al*, 1997).

In yet another method, step iii) is performed by recovering proteins from cells of the organism, separating the recovered proteins and observing a corresponding change in concentration of at least one individual protein. Thus a first aliquot of cells of the organism is cultured under conditions to permit expression of the target gene. Proteins in the cell are recovered and separated e.g. by use of a two dimensional electrophoresis gel. This technique is well known in the literature (Anderson, N L *et al*, 1998) and gives rise to a pattern where the identity of each protein is known or can be determined. Then another aliquot of the cells is incubated under conditions to prevent expression of the target gene. Recovered proteins are separated by electrophoresis to produce a pattern different from the first. The differences are observed and are indicative of genes and regulatory sequences that are involved in a feedback mechanism associated with the target gene.

The first of these three methods involves a substantial capital investment to create a library of constructs of potential regulatory sequences each fused to a

reporter gene; but it should be effective to identify any or all regulatory sequences involved in the feedback mechanism associated with the target gene. The second method using gene expression arrays, avoids the expense of creation of a library, and should be effective to pick up regulatory sequences involved in feedback mechanisms where transcription is altered. The third method, using "proteomics" is relatively simple and cheap, but may not be effective to pick up regulatory sequences where the feed back mechanism involves mRNA synthesis or protein stability or activity.

Use of these methods permits regulatory sequences, involved in feedback regulation of a target gene but which are not promoters of the target gene, to be identified. Once this has been done, it is a simple matter to construct cells as defined, containing an exogenous reporter gene under the control of the regulatory sequence, and to use these cells in a whole cell assay for screening compounds for antibiotic or other biological activity.

The organism is preferably a bacterium e.g. *Bacillus subtilis*, although other prokaryotes and even simple eukaryotes such as yeasts are envisaged. It may be convenient to use an organism whose genome is sufficiently small that it can be chopped up into random fragments of convenient size which constitute a library of potential regulatory sequences, with the number of potential regulatory sequences of the library not being impractically large. This is a property possessed by bacteria and possibly also by yeasts.

For any given target gene, there is thus a good chance that somewhere in the genome there will be a regulatory sequence the activity of which is enhanced or reduced by lack of the target function. In *B. subtilis* it is straightforward to turn off any target gene by making genetic constructions in which the gene can be repressed (e.g. by use of the IPTG-inducible $P_{spac}$ promoter (Yansura & Henner, 1984), or the xylose-inducible promoter, $P_{xyl}$ (Feucht *et al*, 1996)). When the inducer compound is taken away, expression of the gene is blocked and the target function is depleted from the cell. As discussed above, this is likely to result in specific induction of one or more genes. To find the promoter or regulatory sequences for such a gene, a large (>10,000) random collection of short (approximately 50 to 1,000 bp) DNA sequences

from the chromosome of *B. subtilis* or a related organism are fused to an appropriate reporter gene (e.g. *lacZ*). Methods known in the art are available to achieve this (reviewed by Errington, 1990). Each member of the collection of fusions is then introduced into a cell of a strain of *B. subtilis* that had been genetically engineered to allow depletion of the target function. A regulatory sequence exhibiting the desirable property of modulating reporter gene expression upon depletion of the target function would provide a means of screening for compounds which are specific modulators of the target gene function. For example, if the gene encoding one of the subunits of DNA gyrase (*gyrA*) is depleted, the resultant change in DNA supercoiling in the cell should result in increased expression from the promoter of the *gyrA* gene. Some random fragments of DNA (e.g. one containing the *gyrA* promoter itself), when fused to a reporter gene and introduced into the cells in which gyrase has been depleted, would give rise to increased reporter activity. Isolating the gyrA promoter fragment (or some similarly behaving regulatory sequence), and fusing it to a reporter gene, gives a genetic construct that would respond, with increased expression, to inhibition of DNA gyrase by chemical inhibitors (i.e. potential antibiotics) (Menzel & Gellert, 1983). In any operational screen, it is preferable to include a second reporter gene, encoding a non-gyrase-dependent promoter fragment, to control for non-specific inhibitors of gene expression. Alternatively growth of the culture could be followed by, for example measurement of optical density and correction made for non-specific effects on culture growth. In principle, it might sometimes be possible to find, in the random collection, a DNA sequence which, when fused to the reporter and depleted for e.g. the *gyrA* product, would respond with decreased expression. Such a reporter would provide a control reporter that would most likely increase the specifity of the assay for inhibitors of DNA gyrase.

The general strategy may be applicable to other organisms. Yeast genetics should be facile enough to allow it to be used. This opens up the possibility of identifying compounds that inhibit specific eukaryotic functions; at least those that are conserved in yeast. The yeast strain may first have its endogenous gene replaced with the equivalent human gene to make the screen more direct and specific.

**Example 1**

A plasmid (pAT1) containing the N-terminal-coding region of the *gyrA* gene of *B. subtilis* is transformed into *B. subtilis* so as to create strain 2842. In this strain, the plasmid is integrated into the chromosome by homologous recombination, resulting in partial duplication of the *gyrA* gene. The functional copy of *gyrA* that remains has been placed under the control of the repressible $P_{spac}$ promoter, so that removal of IPTG from a growing culture results in depletion of GyrA protein from the cells. To improve the repression of the $P_{spac}$ promoter, a second, autonomously replicating plasmid also was introduced, p65, providing multiple copies of the *lacI* repressor gene (giving strain 2844). GyrA is one subunit of the essential DNA gyrase protein, so that in the absence of IPTG, the culture growth was strongly impaired Fig 1A. Integration of plasmid pAT1 also places a *lacZ* reporter gene under the control of the natural promoter for *gyrA*. The level of expression of this reporter was increased in the absence of IPTG, compared with the control culture in which IPTG remains present (Fig.1B) indicating that the promoter is subject to negative feedback regulation. Reduction in the availability of GyrA protein results in an increase in transcription from the *gyrA* promoter.

In principle, strains 2842 and 2844 provide a means of detecting inhibitors of DNA gyrase, since treatment of the cells with such compounds should similarly elicit increased relative expression of the *lacZ* reporter gene. To demonstrate this, strain 2844 was grown in the presence of IPTG and treated with a range of concentrations of a known inhibitor of DNA gyrase, nalidixic acid. As shown in Fig. 2, at a range of concentrations of nalidixic acid, from 2 $\mu$g/ml through 16 $\mu$g/ml, the specific activity of β-galactosidase (encoded by the *gyrA-lacZ* reporter gene) was increased to about 3-fold greater than that of the untreated cells.

To show that the assay strain could be used to screen compounds for activity against DNA gyrase in high throughput format, samples from a culture of strain 2844 were introduced into the wells of a 96-well microtire plate to which a series of antibiotics with a range of known modes of action had been added. Each antibiotic was added in the form of a 2-fold dilution series. After allowing 2 h for growth, the β-galactosidase specific activity was measured, using culture optical density ($OD_{600}$)

to correct for reductions in cell growth due to the antibiotic action. Figures 3A, B and C plot the activity against the well number. The concentration of antibiotic in well 1 is as follows in $\mu$g/ml Fig 3A: polymyxin: 256, proflavin 165, trimethoprim 128, vancomycin 8, nalidixic acid 128; Fig 3B: ampicillin 128, carbenicillin 1024, chloramphenicol 128, nalidixic acid 128. rifampicin 64; Fig 3C: bacitracin 1024, chlorhexadine 128, monensin 128, novobiocin 128, phosphomycin 750. Wells 2-12 then follow the 2-fold dilution series. As shown in Fig. 3A, B & C, both nalidixic acid and novobiocin stimulated expression from the gyrA promoter in the assay plates, whereas chloramphenicol, kanamycin, rifampicin, carbenicillin, polymyxin B, and vancomycin did not show significantly higher specific activities than the untreated control cells. Interestingly, proflavine and trimethoprim, which affect other aspects of DNA metabolism, also registered as positives in the assay. We conclude that the feedback regulation exploited with the assay strain should be useful in identifying inhibitors of DNA gyrase and possibly compounds acting on other facets of the DNA replication machinery.

**ADDITIONAL REFERENCES**

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CLAIMS

1. A method for identifying a modulator of a bacterial essential protein comprising:

i) providing a bacterial host cell which expresses the essential protein and having a polynucleotide construct comprising a regulatory sequence operably linked to a reporter gene wherein the regulatory sequence is associated with a feed back mechanism responsive to alteration in the synthesis or activity of the essential protein;

ii) contacting a test substance with the host cell; and

iii) monitoring expression of the reporter gene to determine thereby whether the said substance modulates the synthesis or activity of the essential protein.

2. A method according to claim 1 wherein the essential protein is involved in cell wall synthesis, teichoic acid synthesis, DNA replication, RNA synthesis, cell division, chromosome segregation, translation or lipid synthesis.

3. A method according to claim 1 or claim 2 wherein inhibition of the essential protein up-regulates expression of the reporter gene from the regulatory sequence.

4. A method according to any one of claims 1, 2 or 3 wherein the regulatory sequence has the activity of a promoter for the gene encoding the essential protein and inhibition of the essential protein up-regulates expression from its promoter.

5. A method according to any one of claims 1, 2 or 3 wherein the regulatory sequence has the activity of a promoter for a gene which does not encode the essential protein but which is up-regulated via the feedback mechanism in response to alterations to the synthesis or activity of the essential protein.

6. A method according to any one of the proceeding claims wherein the reporter gene comprises a gene which is up-regulated in response to alterations in the synthesis or activity of the essential protein and step (iii) comprises monitoring for differential expression of the gene in the presence or absence of the test substance.

7. A method according to any one of the proceeding claims wherein the bacterial cell is provided with a second polynucleotide construct comprising a promoter operably linked to a second reporter gene and the method further comprises monitoring expression of the second reporter gene.

8. A method according to any one of the proceeding claims comprising determining whether the test substance demonstrates specific inhibition of the essential protein.

9. An inhibitor of a bacterial essential protein identifiable by the method of any one of the proceeding claims.

10. An inhibitor of a bacterial essential protein identified according to the method of any one of claims 1 to 8.

11. An inhibitor according to claim 9 or claim 10 for use in a method of treatment of a human or animal body.

12. An inhibitor according to claim 11 for use as an antibiotic.

13. A pharmaceutical composition comprising the inhibitor of any one of claims 9, 10 or 11 and a pharmaceutically acceptable carrier.

14. A method for identifying a regulatory sequence which is affected by a feedback mechanism on alteration of synthesis or activity of a bacterial essential protein comprising:

i) providing a bacterial cell having a reporter gene under the control of a candidate regulatory sequence;

ii) selecting a target essential protein which is expressed in the organism;

iii) altering the synthesis or activity of the essential protein;

iv) monitoring expression of the reporter gene; and

v) determining thereby whether the candidate regulatory sequence is affected by a feedback mechanism responsive to alteration of the synthesis or activity of the essential protein.

15. A method of identifying a regulatory sequence whose activity is affected by a feedback mechanism or an alteration of the synthesis or activity of an essential bacterial protein comprising:

(a) monitoring expression of a bacterial gene in a bacterial host

cell in the presence of normal and altered synthesis or activity of the essential protein;

(b) identifying differential gene expression in the presence of normal and altered synthesis or activity of the essential protein; and

(c) identifying thereby a regulatory sequence whose activity is affected by the feedback mechanism.

16. A method according to claim 15 wherein step (b) comprises providing an array of nucleotide sequences of the genes of the bacterial cell, recovering polynucleotide material from the cells of the organism and applying such polynucleotide material to the array and monitoring for hybridisation of the bacterial nucleic acid material to the array.

17. A method according to claim 15 wherein step (b) comprises recovering and separating proteins from the bacterial cell and monitoring for a change in concentration of a protein in the presence of normal and altered synthesis or activity of the essential protein.

18. A method according to any one of claims 1 - 8 wherein the regulatory sequence is identified by a method according to any one of claims 14 - 17.

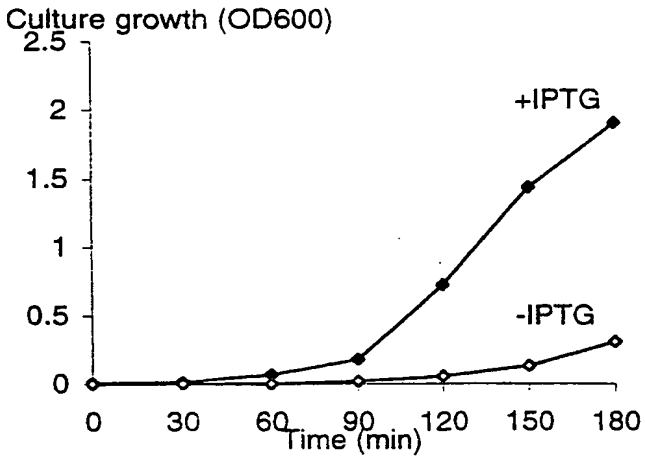


Fig. 1A

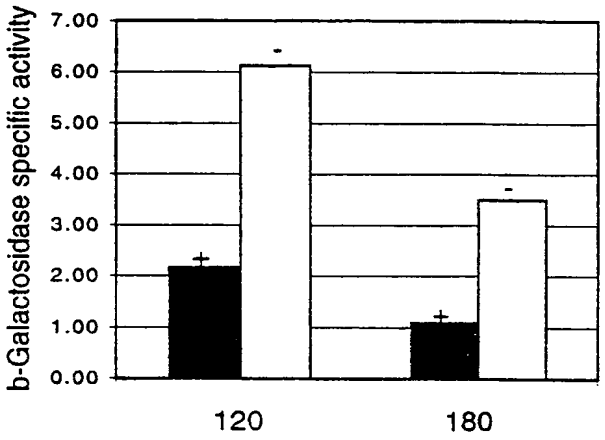


Fig. 1B

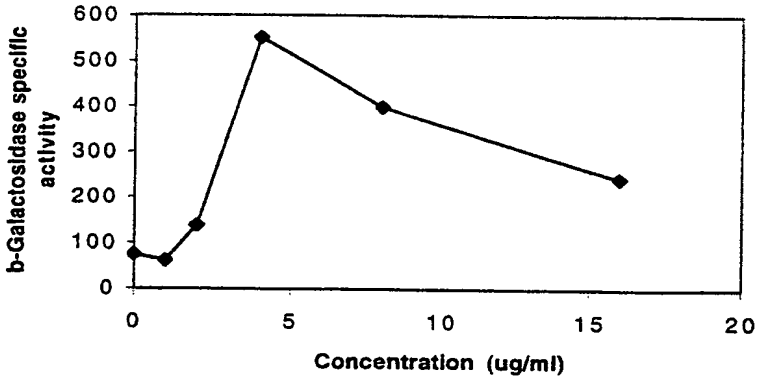


Fig. 2

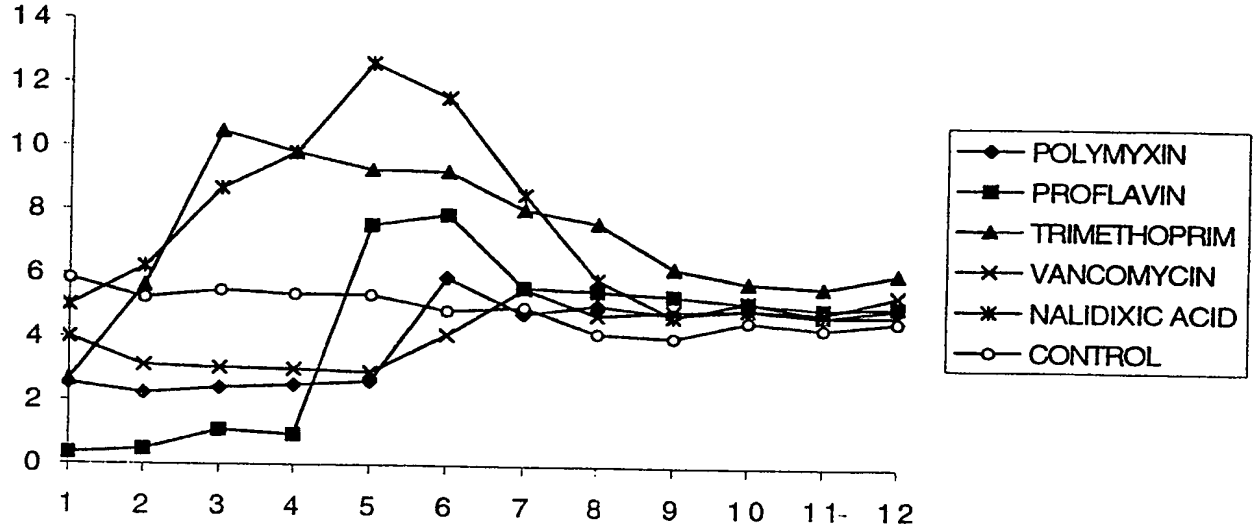
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Fig. 3A

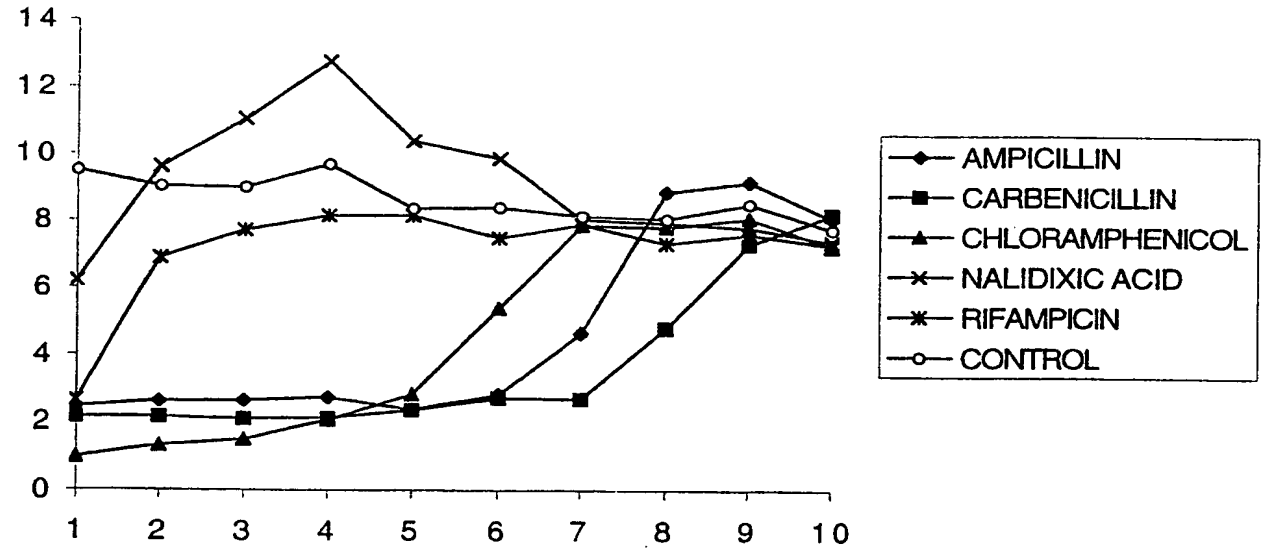
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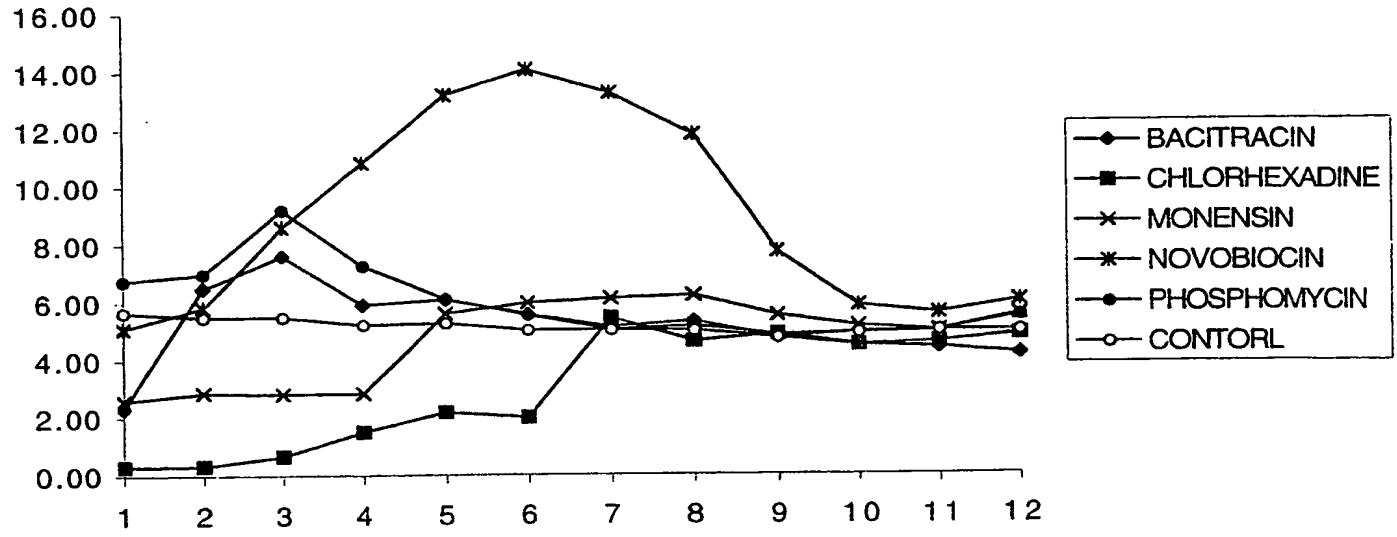
Fig. 3B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CHLORHEXADINE
MONENSIN
NOVOBIOCIN
PHOSPHOMYCIN
CONTORL

Fig. 3C